55. (New) A method according to claim 54, wherein said receptor capable of acting as a silent partner is RXR.

56. (New) A method according to claim 54, wherein said receptor capable of acting as a silent partner is ultraspiracle.

REMARKS

Courtesies extended to Applicants' representatives Stanley H. Kim and Stephen E. Reiter in a personal interview on November 29, 2000 are acknowledged with appreciation.

The present invention provides methods for modulating expression of exogenous genes in cells containing a defined DNA construct. DNA constructs contemplated herein comprise an exogenous gene under the control of a (modified or unmodified) ecdysone response element plus a modified ecdysone receptor which, in the presence of an appropriate ligand, binds to the ecdysone response element, and optionally a further receptor which, in the presence of the modified ecdysone receptor, can act as a silent partner. The invention method comprises providing to a cell containing the construct an effective amount of a ligand for the modified ecdysone receptor that is not normally present in the cell. The presence of ligand for the modified ecdysone receptor (and optionally, the presence of a receptor that can act as a silent partner) promotes the formation of ligand-receptor complexes which can interact with invention modified ecdysone response element, thereby modulating expression of the exogenous gene.

Invention methods for modulating exogenous gene expression are useful in a wide variety of applications. Modulation of exogenous gene expression is desirable in numerous cell populations ranging from transiently modified cells to stably transformed cell lines. For example, invention methods can advantageously be employed in *in vitro* cellular expression systems to regulate expression of a recombinant expression product. Similarly, host cells and other recombinant cell types can benefit from invention methods for modulating the expression of an exogenous gene.

Claims 1 to 46 were pending before this response. By the present communication, claims 25 to 34 and 43 to 46 have been canceled without prejudice. Claims 1, 11, 12 and 20 to 24 have been amended and new claims 47 to 56 have been added in order to define Applicants' invention with greater particularity. No new matter is presented by the amendments or new claims as all new claim language is fully supported by Applicants' specification and original claims. The amendments and new claims do not require a new search or raise new issues for consideration because they address matters previously at issue during prosecution of the subject application. The amendments and new claims are respectfully submitted to place the application in condition for allowance or, in the alternative, to reduce the issues upon appeal. The number of new claims presented does not exceed the number of cancelled claims. Accordingly, entry of the amendments submitted herewith is respectfully requested.

Upon entry of the amendment, claims 1 to 24, 35 to 42 and 47 to 56 will be pending, and these claims are presented for the Examiner's convenience as Exhibit A.

Rejections Under 35 U.S.C. § 112

Applicants respectfully traverse the rejection of claims 1 to 46 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. As discussed in the personal interview, all claims are fully enabled (see, for example, Hoppe *et al.*, (2000) *Mol. Therapy* 1:159-164, provided herewith for the convenience of the Examiner). However, in the interest of facilitating prosecution and reducing the issues, claims 1, 12, and 20 to 24 have been amended to direct the claims in the present invention to *in vitro* methods of modulating gene expression.

Applicants' invention, as defined by amended claims 1, 22, 23, 24, and claims dependent therefrom, requires a method for modulating the expression of an exogenous gene in a cell by providing to the cell an effective amount of a ligand (not normally present in the cell) for a modified ecdysone receptor. The cell contains a DNA construct comprising the exogenous gene under the control of an ecdysone response element and a modified ecdysone receptor which, in

the presence of a ligand (and optionally in the further presence of a receptor that can act as a silent partner of the ecdysone receptor) binds to the ecdysone response element.

Invention methods to modulate the expression of an exogenous gene in a cell are useful in a variety of systems including *in vitro* expression systems (See, for example, page 8, lines 29-32 of Application specification), cellular expression systems (*e.g.*, page 9, lines 20-21), host cells (*e.g.*, page 10, lines 12-14; and page 39, lines 13-16), mammalian expression systems (*e.g.*, page 35, line 33 to page 36, line 1), recombinant cells (*e.g.*, page 42, lines 1-4 and page 43, lines 32-35), and the like. The specification further provides working examples of invention methods in stable recombinant cells lines (see Examples 3 and 6). Expression of an exogenous gene is modulated *in vitro* in stable recombinant cells containing a modified ecdysone receptor, a heterodimeric partner, and an ecdysone inducible report by providing to the cells a suitable ligand such as muristerone or ponasterone.

It is respectfully submitted that the present invention relates to methods for *in vitro* modulation of expression of exogenous genes in cells. In view of the ample support provided in the specification for the *in vitro* embodiments of the present invention, and the amendments to claims 1, 22 to 24 submitted herewith, Applicants respectfully submit that the claims are fully enabled. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph are respectfully requested.

Claim Rejections Under 35 U.S.C. §§ 102 and 103

The rejection of claims 25 to 29 and 31 to 34 under 35 U.S.C. § 102(b) as allegedly being anticipated by Meybeck *et al.* (U.S. Patent No. 5,198,225) is respectfully traversed. This rejection has been rendered most by the cancellation of claims 25 to 29 and 31 to 34.

The rejection of claims 25 to 34 and 43 to 46 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Meybeck *et al.* (U.S. Patent No. 5,198,225) and further in view of Mikitani ((1996) *Biochem, Biophys. Res. Comm.*, 227:427-432) is respectfully traversed. This rejection has been rendered most by the cancellation of claims 25 to 34 and 43 to 46.

In view of the above amendments and remarks, reconsideration and favorable action on all claims are respectfully requested. In the event any issues remain, the Examiner is invited to contact the undersigned at the telephone number set forth below so that a prompt disposition of this application can be achieved.

Respectfully submitted,

Date: 12/13/00

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Enclosures

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In Re Application of:
Evans et al.
Application No.: 09/042,488
Filed: March 16, 1998
Exhibit A: Page 1

PATENT
Attorney Docket No. SALK1520-2

EXHIBIT A: CLAIMS AS THEY WILL STAND UPON ENTRY OF THE AMENDMENT

1. (Amended) A method for modulating the expression of an exogenous gene in a cell containing:

(i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and

(ii) a modified ecdysone receptor which, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising providing to the cell an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in the cell; and wherein said ligand is not toxic to said cell.

2. (Reiterated) A method according to claim 1 wherein said modified ecdysone receptor comprises:

a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain obtained from a DNA-binding protein; and

an activation domain of a transcription factor,

wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor,

with the proviso that when said activation domain is derived from a glucocorticoid receptor, said DNA-binding domain is not derived from a glucocorticoid receptor or an *E. coli* LexA protein.

3. (Reiterated) A method according to claim 2 wherein said modified ecdysone receptor is further characterized as having substantially no constitutive activity in mammalian cells.

4. (Reiterated) A method according to claim 2 wherein the DNA-binding domain of said modified ecdysone receptor is derived from a member of the steroid/thyroid hormone superfamily of receptors.

5. (Reiterated) A method according to claim 2 wherein said activation domain is obtained from a member of the steroid/thyroid hormone superfamily of receptors.

6. (Reiterated) A method according to claim 2 wherein said activation domain is selected from a glucocorticoid receptor activation domain, a VP16 activation domain or a GAL4 activation domain.

7. (Reiterated) A method according to claim 6 wherein said modified ecdysone receptor is selected from VpEcR, VgEcR or GEcR.

8. (Reiterated) A method according to claim 7 wherein said modified ecdysone receptor is VgEcR having the amino acid sequence set forth in SEQ ID NO:5.

9. (Reiterated) A method according to claim 1 wherein said modified ecdysone receptor is present primarily in the form of a homodimer.

10. (Reiterated) A method according to claim 9 wherein said ecdysone response element is the native ecdysone response element.

11. (Amended) A method according to claim 47 wherein said receptor capable of acting as a silent partner is RXR.

12. (Reiterated) A method according to claim 11 wherein said RXR is exogenous to said mammalian cell.

13. (Reiterated) A method according to claim 1 wherein said ecdysone response element is a modified response element which comprises, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

wherein said first half-site has the sequence:

-RGBNNM-,

wherein

each R is independently selected from A or G;

each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

each M is independently selected from A or C;

with the proviso that

at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-; and

said second half-site is obtained from a glucocorticoid receptor subfamily response element.

14. (Reiterated) A method according to claim 13 wherein said response element has substantially no binding affinity for farnesoid X receptor (FXR).

15. (Reiterated) A method according to claim 1 wherein said ligand is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

16. (Reiterated) A method according to claim 15 wherein said naturally occurring ecdysone is α-ecdysone or β-ecdysone.

17. (Reiterated) A method according to claim 15 wherein said ecdysone analog is ponasterone A, ponasterone B, ponasterone C, 26-iodoponasterone A, muristerone A, inokosterone or 26-mesylinokosterone.

18. (Reiterated) A method according to claim 15 wherein said ecdysone mimic is 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazine, an N-substituted-N-alkyl-N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl, carbonyl hydrazine or an N-aroyl-N'-alkyl-N'-aroyl hydrazine.

19. (Reiterated) A method according to claim 1 wherein said exogenous gene is a wild type gene and/or therapeutic gene.

20. (Amended) A method according to claim 19 wherein said wild type gene is selected from genes which encode products:

the substantial absence of which leads to the occurrence of a non-normal state in said cell; or

a substantial excess of which leads to the occurrence of a non-normal state in said cell.

21. (Amended) A method according to claim 19 wherein said therapeutic gene is selected from those which encode products:

which are toxic to the cells in which they are expressed; or

which impart a beneficial property to said cells.

22. (Amended) A method of inducing the expression of an exogenous gene in a cell containing:

(i) a DNA construct comprising an exogenous gene under the control of an ecdysone response element,

(iii) DNA encoding a modified ecdysone receptor under the control of an inducible promoter; wherein said modified ecdysone receptor, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, and

(iii) a ligand for said modified ecdysone receptor;

said method comprising subjecting said cell to conditions suitable to induce expression of said modified ecdysone receptor.

23. (Amended) A method of inducing expression of an exogenous gene in a cell containing a DNA construct containing said exogenous gene under the control of an ecdysone response element, said method comprising introducing into said cell:

a modified ecdysone receptor; and

a ligand for said modified ecdysone receptor,

wherein said receptor, in combination with a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, activating transcription therefrom.

24. (Amended) A method for the expression of a recombinant product detrimental to host cells, said method comprising:

transforming suitable host cells with:

(i) a DNA construct encoding said recombinant product under the control of an ecdysone response element, and

(iii) DNA encoding a modified ecdysone receptor;

growing said host cells in suitable media; and

inducing expression of said recombinant product by introducing into said host cells ligand(s) for said modified ecdysone receptor, and optionally a receptor capable of acting as a silent partner for said modified ecdysone receptor.

35. (Reiterated) A method according to claim 4, wherein said member of the steroid/thyroid hormone superfamily of receptors is selected from: EcR, vitamin D_3 receptor, RARI, RARϑ, RARK, RXRI, RXRϑ, RXRK, TRI, TRϑ, or ER.

36. (Reiterated) A method according to claim 35, wherein the DNA-binding domain of the modified ecdysone receptor is characterized as having a P-box amino acid sequence that differs from the P-box amino acid sequence of the naturally occurring DNA-binding domain.

37. (Reiterated) A method according to claim 36, wherein said modified P-box amino acid sequence preferentially binds to a different hormone response element half-site than said naturally occurring P-box amino acid sequence.

38. (Reiterated) A method according to claim 37, wherein the DNA-binding domain of said modified ecdysone receptor is derived from EcR and the P-box amino acid sequence is GSCKV (SEQ ID NO:3).

39. (Reiterated) A method according to claim 13, wherein said first half-site is obtained from an ecdysone response element and said second half-site is obtained from a hormone response element selected from a glucocorticoid response element, a mineralocorticoid response element, a progesterone response element or an androgen response element.

40. (Reiterated) A method according to claim 39, wherein said first half-site is obtained from an ecdysone response element and said second half-site is obtained from a glucocorticoid response element.

41. (Reiterated) A method according to claim 40, wherein said first half-site is AGTGCA and said second half-site is TGTTCT.

42. (Reiterated) A method according to claim 13, wherein said ecdysone response element has the sequence AGTGCA-N-TGTTCT.

47. (New) A method according to claim 1, wherein said receptor capable of acting as a silent partner is present.

48. (New) A method according to claim 47 wherein said receptor capable of acting as a silent partner is ultraspiracle.

49. (New) A method according to claim 1 wherein said modified ecdysone receptor has substantially no binding affinity for endogenous response elements.

50. (New) A method for modulating the expression of an exogenous gene in a cell containing:

(i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and

(ii) a modified ecdysone receptor which, in the presence of a ligand therefor, and in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising providing to said cell an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in said cell; and wherein said ligand is not toxic to said cell.

51. (New) A method according to claim 52, wherein said receptor capable of acting as a silent partner is RXR.

52. (New) A method according to claim 52, wherein said receptor capable of acting as a silent partner is ultraspiracle.

53. (New) A method for modulating the expression of an exogenous gene in a mammalian cell containing:

(i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and

(ii) a modified ecdysone receptor which, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising providing to said mammalian cell an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in said mammalian cell; and wherein said ligand is not toxic to said mammalian cell.

54. (New) A method according to claim 1, wherein said receptor capable of acting as a silent partner is present.

In Re Application of:
Evans et al.
Application No.: 09/042,488
Filed: March 16, 1998
Exhibit A: Page 9

PATENT
Attorney Docket No. SALK1520-2

55. (New) A method according to claim 54, wherein said receptor capable of acting as a silent partner is RXR.

56. (New) A method according to claim 54, wherein said receptor capable of acting as a silent partner is ultraspiracle.

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ARTICLE

Adenovirus-Mediated Inducible Gene Expression *in Vivo* by a Hybrid Ecdysone Receptor

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Precise control of transgene expression would markedly facilitate certain applications of gene therapy. To regulate expression of a transferred gene in response to an exogenous compound *in vivo*, we modified the ecdysone-responsive system. We combined the advantages of the *Drosophila* (DmEcR) and the *Bombyx* ecdysone receptor (BmEcR) by creating a chimeric *Drosophila*/*Bombyx* ecdysone receptor (DB–EcR) that preserved the ability to bind to the modified ecdysone promoter without exogenous retinoid X receptor (RXR). In cultured cells, DB–EcR effectively mediates ligand-dependent transactivation of a reporter gene at lower concentrations of the chemical ecdysone agonist GS-E than VgRXR (DmEcR + RXR). Transgene delivery *in vivo* was achieved by intramyocardial injection of recombinant adenovirus vectors in adult rats. Upon stimulation with GS-E, DB–EcR potently (>40-fold induction) activated gene expression *in vivo* while VgRXR was not induced. This hybrid ecdysone receptor represents an important new tool for *in vivo* transgene regulation with potentially diverse applications in somatic and germline transfer.

Key Words: ecdysone receptor; *in vivo;* adenovirus; inducible expression; gene transfer.

INTRODUCTION

Expression of foreign genes is used in a wide range of applications including transgenic animals and gene therapy. Given that the expression of most genes is tightly controlled under physiological conditions, precise and predictable regulation of transgene expression in target cells is a logical goal. Regulable expression could provide unique insights into physiological and pathophysiological gene function and may be of practical utility for the development of effective and safe gene therapy. Various inducible principles have been developed for use in cultured mammalian cells (i.e., heat shock, heavy metal ion, tetracycline, steroid, or ecdysone hormone induction) (1–3). For *in vivo* application, tetracycline-, sterold-, rapamycin-, and ecdysone-responsive systems have been studied most intensively in recent times (4–11). The use of ecdysone-inducible transgene expression *in vivo* has several potential advantages over other regulatory systems: ecdysone hormones are not known to affect mammalian physiology (unlike glucocorticoids or progesterone) nor to be toxic or teratogenic (12) (unlike tetracyclines).

Ecdysone hormone responsiveness is mediated by the functional ecdysone response complex, a heterodimer of the insect ecdysone receptor (EcR) either with its natural dimeric partner, the ultraspiracle gene product (USP), or with the retinoid X receptor (RXR), a mammalian homolog of USP (13–16). In most mammalian cells, the *Drosophila* EcR (DmEcR) cannot support high levels of transactivation without supraphysiological levels of RXR (7, 14, 15). In transgenic mice, effective transfer of ecdysone hormone inducibility has recently been achieved by generating mice which express both DmEcR and RXR. These receptor mice were then bred with a reporter mouse line which had an integrated ecdysone-responsive promoter controlling expression of a reporter gene (7) The DmEcR used in these studies contained a modified DNA-binding domain that bound to a hybrid DNA sequence to ensure that there was no cross-reaction with the farsenoid X receptor (FXR) (7). In contrast to DmEcR, the EcR of *Bombyx mori* (BmEcR) is capable of full transactivation with no added exogenous RXR (17). Discrete determinants within the hormone-binding (E) domain and within the hinge (D) domain of BmEcR govern this high-affinity ligand-stimulated RXR interaction. In addition, nonsteroidal ecdysone analogs can more potently activate BmEcR than the DmEcR (17, 18). Chemically derived ana-

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